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Beta vulgaris L. serine proteinase inhibitor gene expression in insect resistant sugar beet

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Abstract Expression pattern of a sugar beet serine proteinase inhibitor gene, *BvSTI*, was characterized in response to mechanical and fall armyworm (FAW; *Spodoptera frugiperda* J.E. Smith) induced wounding. *BvSTI* expression was analyzed in three breeding lines moderately resistant to sugar beet root maggot (*Tetanops myopaeformis* Roder) and in a susceptible line, F1010. Increased mechanical wound induced levels of *BvSTI* expression were observed in all resistant lines as compared to F1010 during the first 24 h. The most intensive response to wounding was observed in one of the resistant lines, F1016, with a maximum 5- and 2.5-fold increase of *BvSTI* transcript levels over non-wounded roots and leaves, respectively. In contrast, slight increase of *BvSTI* transcript levels in leaves and even an initial decrease in roots were observed in F1010. *BvSTI* transcript accumulation in F1016 and F1010 tissues wounded by FAW showed a similar gene expression pattern, but it was delayed and less intense than the response incited by abiotic wounding. On the protein level, BvSTI specific antibody confirmed increased accumulation of the 30 kDa BvSTI protein in wounded leaves but not in roots of F1016 and F1010. Using trypsin inhibition assays, the activity of BvSTI was confirmed in F1016 roots and leaves and F1010 leaves. In F1010 roots BvSTI activity was completely lacking. We conclude that *BvSTI* gene expression was wound induced in the insect resistant germplasm suggesting that *BvSTI* can be used in biotechnological approaches or in breeding programs for improving insect resistance.

Keywords Sugar beet · Serine proteinase inhibitor · Insect resistance

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Introduction

Sugar beet (*Beta vulgaris* L.) is an important food crop, being one of only two plant sources from which sugar is economically produced. Grown in temperate regions of the world, the large succulent taproots of sugar beet are processed into crystalline sucrose that accounts for 35 % of global raw sugar production (Oerke and Dehne 2004; Smith 1987). Planted in the spring and harvested in the autumn of the same year the rosette leaves and the white fleshy taproots are attacked by numerous pests and pathogens that reduce yields by up to 80 % (Jafari et al. 2009; Zhang et al. 2008; Oerke and Dehne 2004; Allen et al. 1985).

Pesticides are only partially effective as they reduce yield losses by only about 26 % (Oerke and Dehne 2004). Targeted alteration of crop genotypes aimed to enhance pest tolerance, mostly by reducing the reproductive rate of a pest, through conventional breeding has produced undesirable effects. Some of these effects, which include reduction of yields, are caused by the transfer of undesirable traits along with the traits of interest. The root yield of an insect resistant breeding line, F1015, was 25 % less than the root yield of commercial hybrids (Campbell et al. 2000). To reduce these negative effects, biotechnological approaches have provided an alternate strategy for germplasm improvement of many important crops (Lemaux 2008; Moose and Mumm 2008). Continued success of biotechnology, however, hinges on the availability of well characterized beneficial genes often derived from valuable germplasm used in breeding programs.

One of the major objectives of sugar beet breeding programs is the identification of quantitative trait loci (QTL) or particular genes that can be used for improving disease and insect resistance (Butorina and Kornienko 2011; Collard et al. 2005; Weber et al. 2000). To focus on sugar beet DNA loci important in insect resistance, genes incited by the most destructive insect pest of sugar beet in North America, the sugar beet root maggot (SBRM, *Tetanops myopaeformis* Roder), were identified and characterized (Campbell et al. 2008; Smigocki et al. 2008; Puthoff and Smigocki 2007; Campbell et al. 1998). SBRM is found in more than half of all North American sugar beet acreage and causes seedling wilt and death, secondary root growth, reduced taproot size and secondary pathogen invasions, all leading to significant crop damage and yield loss. To date, only three sugar beet lines, F1016, F1015 and F1024, with moderate but incomplete levels of resistance to SBRM have been released for use in sugar beet improvement programs (Campbell et al. 2000, 2010). Gene identification study on SBRM-infested roots of a line reported to have the highest level of SBRM resistance, F1016, and a susceptible F1010 line revealed more than 150 root genes whose activity was regulated by SBRM larval feeding (Puthoff and Smigocki 2007; Smigocki et al. 2006). One of the identified genes encodes a Kunitz-type serine proteinase inhibitor, *BvSTI*, belonging to a class of proteinase inhibitors (PIs) that are involved in hydrolytic deactivation of trypsin. PIs are attractive tools for crop improvement because of their significant protective role in natural defense mechanisms against herbivore insects (Fan and Wu 2005; Lawrence and Koundal 2002; Ussuf et al. 2001). The defensive capacities of plant PIs rely on inhibition of proteinases present in insect guts, causing a reduction in the availability of amino acids necessary for their growth and development (De Leo et al. 2002). Constitutive and temporal expression of recombinant PI genes in transgenic plants enhanced insect and nematode tolerance (Schlüter et al. 2010; Ninković et al. 2007; Samac and Smigocki 2003; Telang et al. 2003). More than 80 % of *Helicoverpa armigera* gut serine proteinases were inhibited with bitter gourd PIs in transgenic plants (Telang et al. 2003). Similarly, artificial diet laced with the gourd PIs reduced larval and pupal weights by about 43 and 26 %, respectively, and had a deleterious effect on fecundity of *H. armigera* larvae. Several coleopteran pests that commonly use cysteine proteinases for protein digestion, as well as nematodes, were inhibited by cysteine PI genes of the *oryzacystatin I* and *II* gene family (Schlüter et al. 2010; Pandey and Jamal 2010; Ninković et al. 2007; Samac and Smigocki 2003; Urwin et al. 1995; Kondo et al. 1990; Abe and Arai 1985). In addition to insect attack, mechanical wounding and microbial infection also augment PI levels (Turra et al. 2009; Pearce et al. 1993).

Serine proteinases make up the major class of digestive enzymes in SBRM larval guts that were inhibited by a soybean trypsin (serine type) inhibitor (Wilhite et al. 2000). These findings suggest that the serine proteinase inhibitor gene *BvSTI* cloned from line F1016 could potentially enhance SBRM resistance and be used for incorporating insect resistance into agronomically important crops. To design effective strategies for using the *BvSTI* gene in molecular breeding programs, a better understanding of its *in planta* regulation is needed. In this study, we compared the expression pattern of *BvSTI* gene in lines F1016 and F1010 in response to mechanical and herbivore induced wounding. *BvSTI* transcript levels and BvSTI protein accumulation and activity were analyzed in wounded roots and leaves. *BvSTI* transcripts in two additional lines, F1015 and UT-8, known to provide some root maggot resistance were compared to F1016 to confirm any association between the expression pattern and resistance.

Materials and methods

Plant material and insects

Sugar beet breeding lines F1016, F1015 and UT-8 with varying levels of moderate resistance to SBRM and a susceptible line, F1010, were used in this study (Campbell 1990; Campbell et al. 2010). Seeds were germinated overnight at room temperature in the dark and then planted in Pro-Mix (Profesional Horticulture) and Perlite soil mixture. Seedlings were grown in the growth chamber at 25 °C during the day and 18–20 °C at night with a day length of 16 h. After 4 weeks, seedlings were transferred to the greenhouse and maintained at 20–30 °C during the day and 18–25 °C over night with a day length of 14–16 h. Plants were fertilized monthly with Osmocote (ScottsMiracle Gro, Marysville, OH). Gene activity analyses were done on tissues collected from 6 week and 3, 4 and 6 month old plants. Protein analysis was done on 6 month old plants.

For insect wounding experiments, fall armyworm (FAW, *Spodoptera frugiperda* J.E. Smith; Benzon Research, Carlisle, PA), a generalist herbivore that feeds on sugar beet leaves, was used as an insect model. Newly emerged larvae were reared on an artificial diet of agar, wheat germ-casein (Product# F0635, Benzon Research), vitamin supplements (Product# F0717, Benzon Research) and neomycin sulfate. Insects were fed daily with fresh diet.

Mechanical and insect wounding

Sugar beet leaves were mechanically wounded by making two to four 1 cm incisions on each side of the leaf, avoiding the mid-veins (Fig. 1a). Roots from 6 week old seedlings were pinched with forceps at 5 mm intervals over the entire root length (Fig. 1b), while older taproots were wounded on the surface by making 5 mm incisions 2–3 mm deep along the entire root length (Fig. 1c). All tissues were maintained on water moistened filter paper. Samples were collected

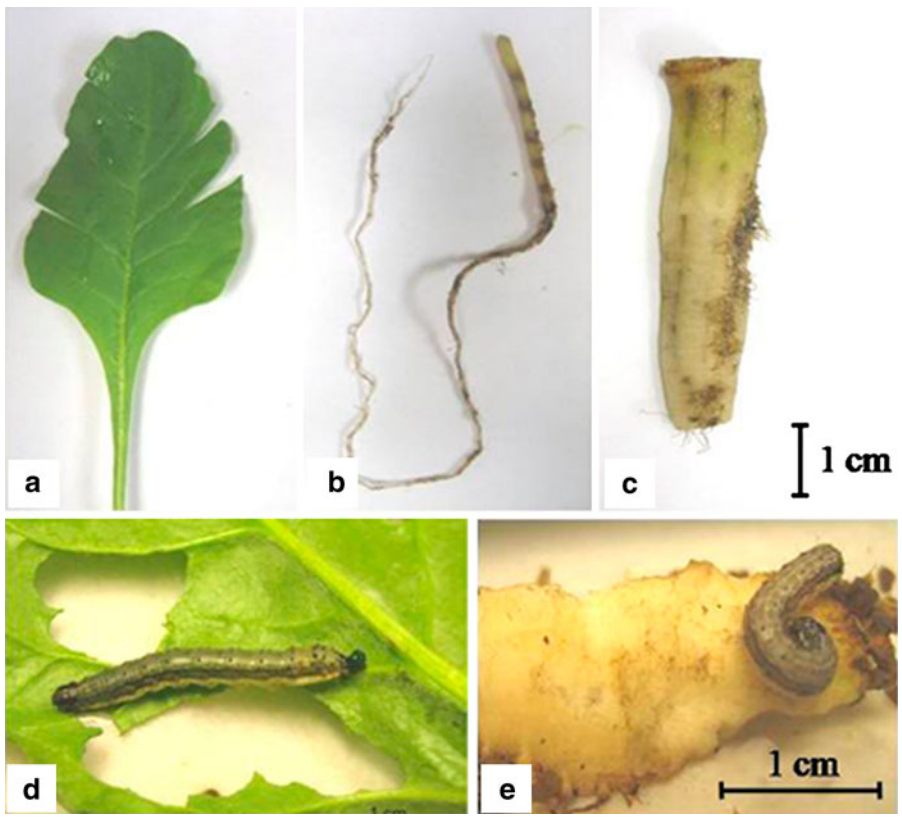

Fig. 1 Wounding of F1010 sugar beet leaves and roots. Mechanical wounding of leaves (**a**), seedling roots (**b**) and 6 month old roots (**c**). FAW larvae feeding on leaves (**d**) and roots (**e**) of 6 month old F1010 plants

prior to wounding (zero time point) and at 2, 6, 24, 48 and 72 h after wounding and stored at −80 °C for analysis. For each time point two biological replicates, tissues from two individual plants, were used.

Ten day old FAW larvae starved for 2 h prior to start of the feeding experiment were used to infest and wound F1016 or F1010 leaf and root tissues (Fig. 1d, e). Each leaf or root collected from 4 month old plants was infested with a single larva. Samples were collected over a 72 h period as above. Two biological replicates, as above, for each time point were generated.

RT-PCR analysis

Total RNA treated with DNAse was isolated using an RNeasy Plant Mini Kit (Qiagen). An 0.6 kb fragment of the *BvSTI* gene transcript was amplified using 100 ng of the total RNA and *BvSTI* gene specific primers (forward 5′-ACCATGGCTTCCATTTTCCT-GAAATC-3′ and reverse 5′-GGTCACCTAGAC-CATCGCTAAAACATCA-3′; (Titanium One-Step RT-PCR Kit, Clontech Laboratories; Smigocki et al. 2008). Following conditions were used for RT-PCR amplification: 50 °C for 1 h, 94 °C for 2 min 40 s, 35 cycles of 94 °C for 20 s, 56 °C for 40 s, 72 °C for 1 min 30 s, followed by 72 °C for 5 min. Transcripts of the constitutively expressed plant *actin* gene (0.54 kb fragment) were used as loading controls to normalize RT-PCR results. The following *actin* primers were used (forward 5′-GTATTGTKAGCAACTG GGATGA-3′ and reverse 5′-AACKYTCAGCCC RATGGTAAT-3′) and performed under the same conditions as described above. Negative controls included samples with no RNA template. RT-PCR products were analyzed by electrophoresis on 1.2 % agarose gels. The quantification of gene expression was done by densitometry, using the ImageJ software ver. 1.32j, NIH, USA. Transcript levels were normalized to the transcript level of the *actin* control run with the same RNA for each sample and presented as percentage of the *BvSTI* relative to the *actin* transcript. Comparison of *BvSTI* transcript levels were based upon the zero time point of F1016 leaves set at 100. RT-PCR analyses were repeated two times with comparable results.

Protein extraction

Total proteins from mechanically wounded leaves and roots of 6 month old F1016 and F1010 sugar beet lines were extracted in ice cold 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM EDTA, 10 % sucrose, 10 mM ascorbic acid, 1 mM PMSF, 2 mM DTT (Smigocki et al. 2009a; Wang et al. 2003; Chan and DeLumex 1982). Tissues were ground in liquid nitrogen and cold extraction buffer was added in proportion of 10 ml per 1 g of tissue. Plant extracts were subsequently cleared by centrifugation at 10,000 rpm for 10 min and the supernatant (crude extract) was concentrated to about 1 ml using Amicon Ultra 15 (3 K) concentrator (Millipore) at 4 °C. The concentrated extract was desalted in 8.5 ml of 62.5 mM Tris-HCl pH 6.8 two times and centrifuged until the retentate volume was less than 200 µl. Total proteins were quantified according to Bradford (1976).

Western blots

Sugar beet total proteins (15 or 30 µg) were separated on 12 % SDS-PAGE gels in 0.025 M Tris, 0.192 M glycine and 3.5 mM SDS running buffer. After electrophoresis, gels were equilibrated in cold transfer buffer (0.025 M Tris, 0.192 M glycine, 0.025 % SDS) for 1 h. Separated proteins were subsequently transferred to Immun-Blot PVDF Membranes (0.2 µm, BioRad) for 1 h 20 min at 70 V (Bio-Rad Mini-Trans-Blot Electrophoretic Transfer cell). Following transfer, membranes were rinsed in deionized water and gently agitated in blocking solution (5 % BLOT-QuickBlocker, Chemicon International) for 1 h and then incubated with rabbit anti-BvSTI antibodies (GenScript Corporation, NJ, USA) diluted 1:1,000, 1:2,000 or 1:5,000 (v/v) in 1 × TBS-T (0.137 M NaCl, 0.02 M Tris pH 7.6, 0.1 % Tween 20). After 1.5 h incubation, membranes were rinsed two times in 1 × TBS-T for 10 min each, and incubated for 1 h in alkaline phosphatase conjugated secondary antibody (AP Conjugated Goat anti-Rabbit IgG, 1:5,000 diluted in 1 × TBS-T, Chemicon International). Membranes were washed in 1 × TBS-T two times for 15 min and then 1 min in 1 × TBS to remove the Tween 20. Alkaline phosphatase was detected using BCIP/NBT (5-bromo-4-chloro-30-indolylphosphate p-toluidine salt and nitro-blue tetrazolium chloride, respectively, Roche) in 0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, and 0.05 M $MgCl_2$ (Smigocki et al. 2009b). The relative levels of the accumulated BvSTI proteins were estimated by determining band density using Image-Quant software (ver. 5.2, Molecular Dynamics, Sunnyvale,

CA). Level of accumulated BvSTI protein was presented relative to the level in F1016 leaf sample collected prior to wounding (zero time point) set as 100. Experiments were repeated and similar results were obtained.

In-gel trypsin inhibitor activity analysis

Native proteins (15 μg) of non-wounded and mechanically wounded roots and leaves were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). For trypsin proteinase activity analyses, gels were incubated with gentle shaking in 25 % (v/v) 2-propanol, 10 mM Tris-HCl pH 7.4 for 30 min to remove SDS followed by 10 mM Tris-HCl pH 8.0 for another 30 min to renature the proteins (Smigocki et al. 2008; Cai et al. 2003; Wang et al. 2003). Gels were than soaked in 40 μg/ml bovine trypsin (Sigma) in 50 mM Tris-HCl pH 8.0, 50 mM $CaCl_2$ for 40 min. Subsequently, trypsin digestion was performed in freshly prepared substrate-dye solution consisting of 2.5 mg/ml *N*-acetyl-DL-phenylalanine *β*-naphthyl ester (Sigma) resuspended in dimethylformamide and 0.5 mg/ml tetrazotized *O*-dianisidine (Sigma) resuspended in 50 mM Tris-HCl pH 8.0 with 50 mM $CaCl_2$ for 30 min at room temperature. Acetic acid (10 %) was added to stop the reaction. Clear zones corresponding to proteins with trypsin inhibitory activity were recorded. *Nicotiana benthamiana* plants transformed with the 35S-*BvSTI* gene construct were used as a positive control for the *BvSTI* encoded proteinase inhibitor activity (Smigocki et al. 2008). Analyses were repeated two times with comparable results.

Results

BvSTI gene expression in mechanically wounded F1016 and F1010 tissues

Mechanical wounding of leaves and roots affected the *BvSTI* transcription in both lines over a 72 h period (Fig. 2a, b). In both F1016 and F1010 a distinct transcription pattern was observed that was similar at each vegetative stage, i.e. 6 weeks, 3 months or 6 months, within each germplasm. Highest levels of *BvSTI* transcripts accumulated in 6 month old plants.

BvSTI transcription in 6 month old tissues was up-regulated by mechanical wounding in F1016 roots and leaves, as well as in F1010 leaves, while in F1010 roots *BvSTI* transcription was down-regulated over the first 24 h following wounding.

In F1016 roots and leaves (Fig. 2a), strong induction of transcription was observed up to 48 h after wounding. Highest levels of root *BvSTI* transcripts were recorded during the first 24 h after wounding with more than a 4-fold increase at 2 and 6 h and 3.5-fold at 24 h, as compared to roots collected prior to wounding. At 48 and 72 h, F1016 root transcript levels were reduced but still slightly above those at the zero time point. In the F1016 leaves, transcripts increased about 2-fold at 2, 24 and 48 h with highest increase of more than 2.5-fold at 6 h. Transcription decreased to basal, non-wounded tissue levels at 72 h.

In contrast to the 3.5 to 4-fold increase observed in F1016 roots, F1010 root *BvSTI* transcript levels were down-regulated by wounding at 2 h and that decrease was more pronounced over the next 24 h (Fig. 2b). At 6 and 24 h time points, the F1010 transcript levels were about 10-fold lower than those in F1016. At 48 and 72 h, transcript levels increased slightly over those in non-wounded tissues (0 time point) and were similar in both lines. In F1010 leaves, the highest accumulation of *BvSTI* gene transcript products was observed at 2 h after wounding followed by a steady decrease to near basal levels. The 1.7-fold increase detected at 2 h was still more than two times lower than in the resistant F1016 line. The reduction in transcripts in F1010 was even more considerable at the 6, 24 and 48 h after wounding reaching about a 5-fold decrease at 48 h as compared to the F1016 leaves.

Western blot detection of BvSTI protein

Proteins of about 30 kDa cross-reacted with the BvSTI antibodies in both F1016 and F1010 leaf samples. Following mechanical wounding, consistently higher levels of BvSTI proteins accumulated in F1016 leaves as compared to F1010 (Fig. 3). Before wounding, basal levels of protein in both lines were similar. However, 2 h after wounding, the amount of BvSTI protein in F1016 was almost doubled, while the F1010 BvSTI accumulation was close to the basal level. F1016 BvSTI protein levels remained elevated over a 72 h period with specific up and down pattern with an approximate 1.8-, 2.5- and 2.4-fold increase observed at 2, 24 and 72 h, respectively (Fig. 3a). The accumulation at 6 and 48 h from wounding were slightly increased over basal level. In F1010, a 1.7-fold increase was detected at 6 h after

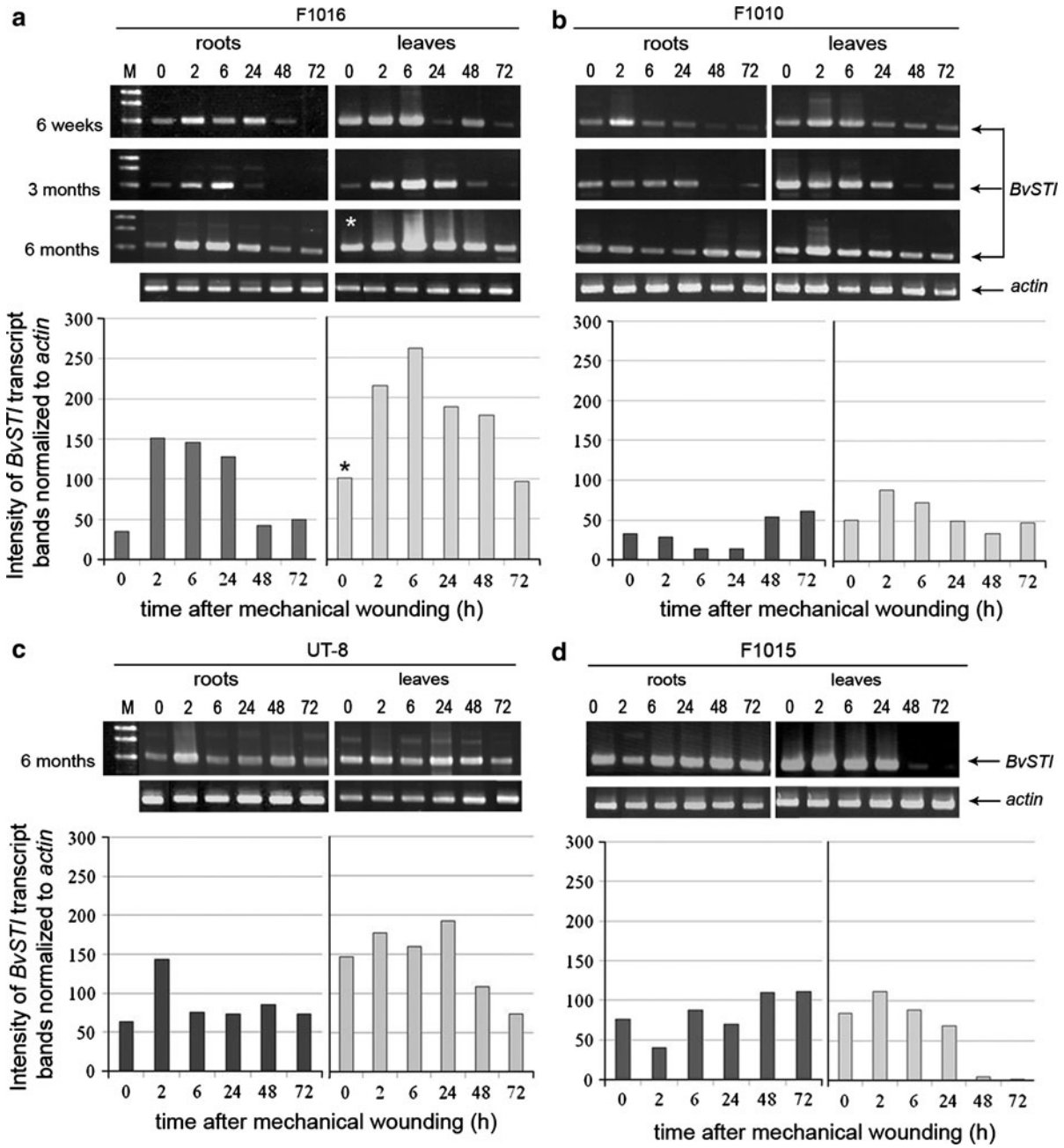

Fig. 2 *BvSTI* gene transcription patterns in sugar beet germplasm (F1016, F1010, UT-8 and F1015) were analyzed by RT-PCR. Root and leaf samples were screened for *BvSTI* gene transcripts (0.6 kb) prior to wounding (zero time point) and at 2, 6, 24, 48 and 72 h after mechanical wounding. *BvSTI* gene transcript levels in SBRM-resistant F1016 (**a**) and susceptible F1010 line (**b**) were compared at several stages of vegetative growth (6 week, and 3 and 6 month old plants). *BvSTI* gene expression analysis in two additional sugar beet lines with reported lower levels of resistance, UT-8 (**c**) and F1015 (**d**) was done on 6 month old tissues. Plant *actin* gene transcripts (0.54 kb) were run for each analyzed sample to normalize the amounts of accumulated *BvSTI* transcripts. The 6 month old *BvSTI* F1016 leaf zero time point (*) was set at 100 for the normalization. *Each bar* represents the relative amounts of *BvSTI* transcripts obtained from one of two independently performed experiments with comparable results. M, Lambda *Hin*dIII molecular mass marker

wounding while all the other time points were similar to the basal non-wounded level (Fig. 3b).

No cross-reacting proteins were observed in any of the F1016 or F1010 root samples at protein concentrations twice (30 μg) those used for analysis of the leaves (data not shown).

BvSTI serine proteinase inhibitor activity

Multiple clear zones (white bands) representing proteins with trypsin inhibitor activity were detected in both lines before and after wounding of root and leaf tissues (Fig. 4). Up to four clear zones ranging in size from approximately 30–50 kDa in F1016 and 30–36 kDa in F1010 were visualized. The observed 30 kDa protein band corresponds to the *BvSTI* gene encoded inhibitor. A 50 kDa trypsin inhibitor protein was detected in F1016 tissues but was not present in any of the analyzed F1010 samples.

Trypsin inhibitor activity in non-wounded F1016 roots was relatively high as compared to the F1010 roots (Fig. 4). Wounding F1016 roots resulted in an overall increase in all of the observed trypsin inhibitor activities (30–50 kDa). Wounding of F1010 roots increased inhibitor activity of a single 36 kDa protein. Leaf basal inhibitor activity in F1016 (30–50 kDa) and F1010 (30–36 kDa) was weak but overall slightly higher in F1010. Wounding of the leaves increased

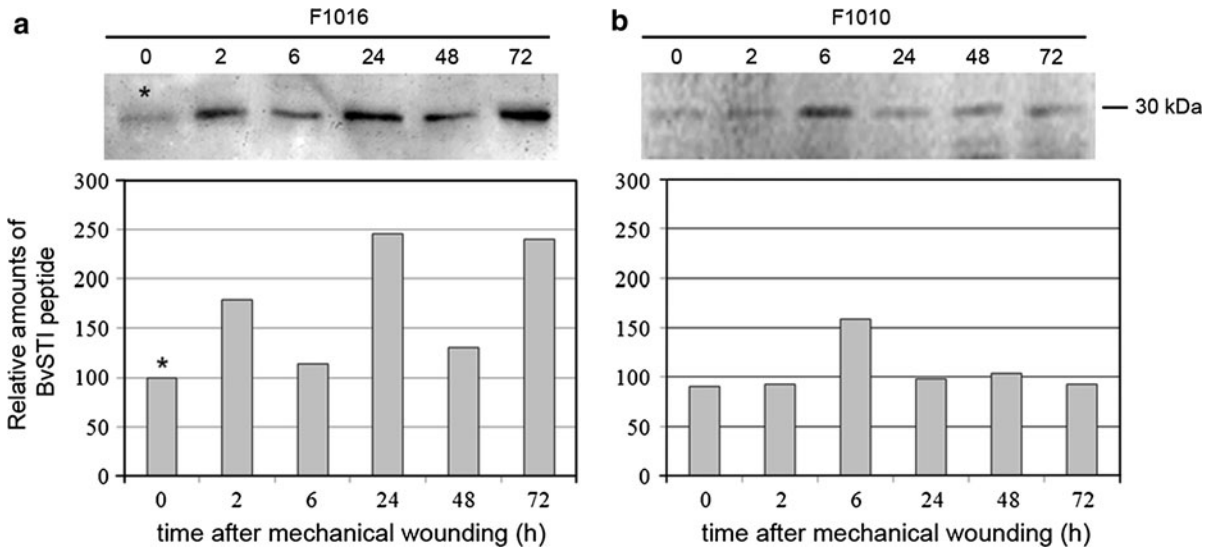

Fig. 3 The wounding-induced accumulation of BvSTI protein in leaves of 6 month old F1016 (**a**) and F1010 (**b**) germplasm. Expression of BvSTI was examined by immunoblot analysis in non-wounded (zero time point) leaves and at 2, 6, 24, 48 and 72 h after mechanical wounding. The relative levels of BvSTI were estimated by determining band density using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Amounts of accumulated BvSTI proteins in all samples were presented relative to the level of BvSTI in non-wounded F1016 which was set as 100 (*)

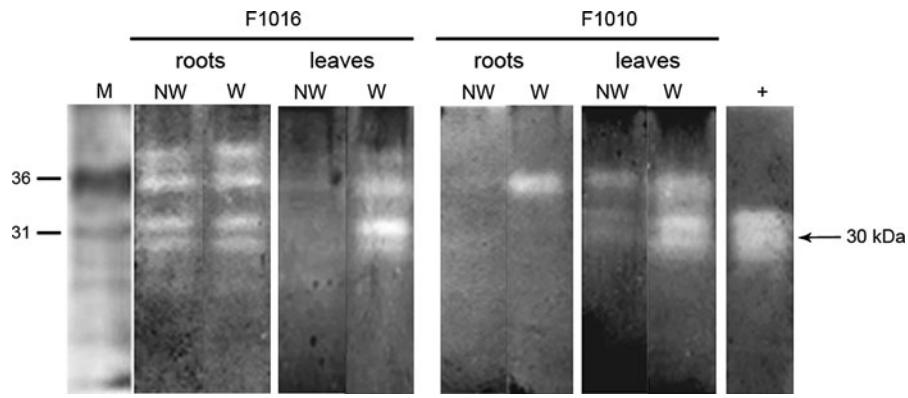

Fig. 4 Activity of BvSTI protein in mechanically wounded 6 month old F1016 and F1010 germplasm. In-gel trypsin inhibitor activity in roots and leaves was analyzed by native-PAGE in non-wounded (NW) and wounded (W) tissues. M, protein molecular mass marker; +, positive control, BvSTI recombinant protein

trypsin inhibitor activities in both lines that included the 30 kDa BvSTI protein.

FAW induced *BvSTI* gene expression

When FAW larvae were fed F1016 and F1010 roots, a low level of *BvSTI* gene transcript induction was observed in both lines (Fig. 5). Root transcripts increased about 2- and 1.5-fold in F1016 and F1010, respectively, at 72 h (Fig. 5). Leaf transcript levels were in general higher in F1016 than the F1010 before and after FAW feeding over a 24 h period. F1016 basal levels before wounding were 1.5 times those of F1010. FAW induced accumulation of *BvSTI* mRNA continued to increase slightly at 2, 6 and 24 h (1.1, 1.2 and 1.3-fold increase, respectively) over basal levels as compared to 6 h in F1010 when they increased 1.5-fold. The highest F1010 transcript level detected at 6 h was lower than any of the F1016 levels at 0–24 h. At 48 and 72 h, transcript levels decreased to relatively low levels in both lines.

BvSTI gene expression in mechanically wounded UT-8 and F1015 tissues

The UT-8 line used in breeding programs for incorporating SBRM resistance into F1016 exhibited *BvSTI* gene activity much like that of F1016 (Fig. 2c). Maximum accumulation of *BvSTI* transcripts after wounding was observed at 2 h in roots and 2 and 24 h in leaves. In contrast to F1016, UT-8 root expression at 6, 24, 48 and 72 h returned to near basal levels prior to wounding. Almost 1.5 and 3 times more *BvSTI* mRNA accumulated in non-wounded UT-8 leaves than in F1016 and F1010, respectively (Fig. 2a, b), highest of any of the analyzed lines. Wounding induced a slight increase in *BvSTI* transcripts over a 24 h period followed by a decrease at 48 and 72 h (Fig. 2c).

BvSTI gene expression patterns in the F1015 line with registered SBRM resistance had similarities to the susceptible F1010 line (Fig. 2d). In roots, wounding initially down regulated *BvSTI* expression at 2 h followed by a return to near non-wounded levels at 6 and 24 h and a 1.6 increase at 48 and 72 h. In F1015 leaves, basal gene transcript levels at the zero time point were similar to F1016. Highest transcript levels were detected 2 h after mechanical wounding, representing a 1.3-fold increase that was still below the levels induced in F1016 and UT-8. At 48 and 72 h after wounding, leaf *BvSTI* transcripts were reduced to levels below those in the F1010 susceptible line.

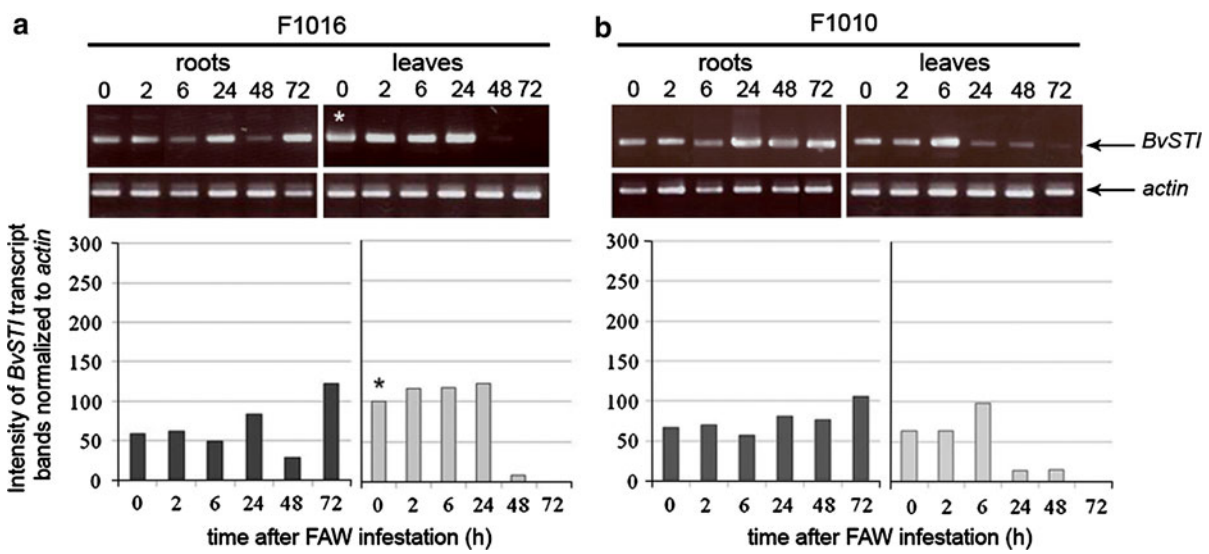

Fig. 5 Insect feeding-induced *BvSTI* gene expression in F1016 (**a**) and F1010 (**b**) germplasm. RT-PCR analysis was done on roots and leaves collected prior to wounding (zero time point) and at 2, 6, 24, 48 and 72 h after FAW wounding. Accumulation of *BvSTI* transcripts was normalized to the plant *actin* gene as in Fig. 2 above

Discussion

Strategies for insect pest protection include transgenic crops that produce products with insecticidal properties (Lemaux 2008; Lawrence and Koundal 2002). Proteinase inhibitor (PI) genes are among the prime candidates suitable for crop improvement since they inhibit digestive enzymes in insect midguts (Dunse et al. 2010; Maheswaran et al. 2007; Abdeen et al. 2005). Over-expression of PI genes significantly reduced or inhibited larval growth and feeding on transgenic plants. In a reciprocal experiment where the expression of a PI gene was suppressed in transgenic potato, an increase in larval weights of Colorado potato beetle (*Leptinotarsa decemlineata)* and beet armyworm (*Spodoptera exigua*) was reported (Ortego et al. 2001). To target the vast number of insect pests, new PI genes need to be identified and characterized. Expression patterns of a sugar beet PI gene that codes for a Kunitz-type trypsin inhibitor (*BvSTI*; Puthoff and Smigocki 2007) was investigated in this study. *BvSTI* transcription was analyzed in several sugar beet lines with enhanced SBRM resistance. Of the three resistant lines analyzed in this study (F1016, F1015 and UT-8), F1016 was reported to have the highest level of SBRM tolerance (Campbell et al. 2000). Since PI genes are generally wound-inducible (Telang et al. 2003; Pearce et al. 1993), we evaluated *BvSTI* gene expression in response to mechanical wounding and FAW larval feeding. Overall, higher wound induced levels of *BvSTI* gene expression were observed in the SBRM resistant lines over the first 24 h following mechanical wounding as compared to the susceptible F1010 line (Fig. 2). However, the highest levels of *BvSTI* transcripts were observed in F1016 where noticeable gene induction and transcript accumulation was evident over the first 24 h after wounding in roots and 48 h in leaves (Fig. 2a). The UT-8 line with less SBRM resistance exhibited a similar induction pattern but with lower levels of accumulated *BvSTI* mRNA, especially in roots (Fig. 2c). However, the third resistant line, F1015, mimicked a contrasting pattern of expression that was more similar to the susceptible F1010 roots as mechanical wounding induced an initial down-regulation of *BvSTI* expression at 2 h (Fig. 2b, d). However, unlike F1010 where the down regulation continued over the next 24 h, F1015 root transcripts returned to near basal levels. Different expression patterns of *BvSTI* gene in these resistant lines may be attributed to their different origin. The F1016 line was derived from the original resistant UT-8 line and in our studies exhibited a similar *BvSTI* induction pattern. On the other hand, F1015 was derived from susceptible F1010 populations by mass selection for SBRM resistance (Campbell et al. 2000), and therefore, may exhibit some F1010 characteristics, i.e. initial down regulation of expression of the *BvSTI* gene in response to wounding. In field trials, SBRM damage ratings (on a zero to nine scale) were between 1.8 and 2.6 for F1016 as compared to 3.1–3.6 for F1015 and 4.7–5.8 for commercial susceptible hybrids with no insecticide application (Campbell et al. 2000). The observed *BvSTI* gene transcription patterns are in line with the reported field trial damage data. Induction of expression in the resistant lines, especially in F1016, suggests active synthesis of defense PI proteins as a response to wounding damage.

Although mechanical wounding is commonly used to investigate PI gene induction patterns, herbivore induced changes in PI gene expression may be distinct from those induced by mechanical wounding (De Vos et al. 2005; Korth and Dixon 1997). Therefore, insect induced *BvSTI* gene expression was evaluated in F1016 and F1010, two lines with the most divergent levels of reported SBRM resistance. Since SBRM larvae cannot be reared in the laboratory and must be collected from infested fields during the growing season, we chose another pest of sugar beet, a general herbivore FAW, for analysis of insect induced *BvSTI* gene expression. FAW feed on sugar beet leaves and also on sugar beet hairy roots (Smigocki, unpublished). Although FAW belong to another order of insects (*Lepidoptera*) and have different feeding habits than SBRM, they like SBRM utilize serine proteinases as their major midgut digestive enzymes (Srinivasan et al. 2006) thus providing a model system for investigating the potential inhibitory role of the BvSTI protein on serine gut proteinases. Previous studies have shown that FAW larvae that were fed transgenic tobacco leaves or sugar beet hairy roots that express the *BvSTI* transgene exhibited higher mortality rates or were delayed in growth and development relative to control larvae (Smigocki et al. 2009a, b). In this study, we demonstrated that *BvSTI* transcripts accumulated in response to FAW feeding in both the resistant and susceptible line, but the response was distinctly reduced in F1016 as compared to mechanical wounding (Figs. 2a, 5). In F1016 leaves, FAW infestation

caused only a 1.2-fold increase in transcripts as compared to 2.5 to 4-fold increase due to mechanical wounding during the first 24 h. An almost complete absence of mRNA in leaves was seen at 48 and 72 h in F1016 and at 24, 48 and 72 h in F1010, likely due to the fact that the tissue was heavily damaged by the larvae at that point. In roots, FAW wounding induced a similar *BvSTI* gene expression pattern in both lines characterized by a slight up and down pattern during the first 24 h followed by a maximum (about 2-fold increase) at 72 h after infestation. The variation in *BvSTI* expression patterns observed in response to wounding or insect feeding are similar to what has been reported by others, i.e. many genes that were strongly induced by mechanical damage were less or not at all induced by larval feeding (Reymond et al. 2000).

At the protein level, in addition to the expected 30 kDa *BvSTI* gene encoded protein, multiple trypsin inhibitor activities were detected in the molecular mass range of about 30–50 kDa in F1016 and 30–36 kDa in F1010 (Fig. 4). Existence of PIs with physiological roles other than protection against the proteolytic enzymes of parasites and insects is widespread in plants (Fan and Wu 2005). PIs are involved in many processes such as regulation of endogenous proteinases during seed dormancy or reserve protein mobilization (Fan and Wu 2005; Brzin and Kidric 1995). Among the pool of trypsin inhibitor activities in F1016 and F1010, BvSTI specific antibodies cross-reacted with a 30 kDa leaf protein (Fig. 3), giving a specific pattern of protein accumulation in mechanically wounded leaves. This pattern differed from the *BvSTI* gene transcription pattern in both analyzed lines. The accumulation of gene transcripts in F1016 and F1010 line was highest at 6 and 2 h after wounding, respectively (Fig. 2a, b). Protein accumulation, on the other hand, peaked at 24 and 6 h in F1016 and F1010, respectively. Lack of direct correlation between transcription and protein activity or accumulation has been reported by others. Searching for genes responsible for elevated nematode resistance in sugar beet, Cai et al. (2003) found that inhibition of nematode development correlated with sporamin PI activity, but not with the amount of inhibitor accumulated in *Agrobacterium rhizogenes*-transformed hairy roots expressing the sporamin *SpTI-1* gene. Similarly, Wu et al. (1997) demonstrated in transgenic potato plants much higher increases of a recombinant extracellular anionic peroxidase using Western blots than by Northern analysis of gene activity. The transgenic potato had a 6- to 20-fold increase of peroxidase protein whereas only a 2- to 6-fold induction of mRNA transcripts was observed on Northern blots. The accumulated F1016 leaf BvSTI protein remained at a relatively high level up to 72 h after mechanical wounding (Fig. 3a). In contrast, Western analysis of F1010 revealed only a single time point at 6 h where the BvSTI protein level was elevated over the same 72 h period (Fig. 3b). These findings suggest that the higher levels of accumulated BvSTI protein detected in F1016 wounded leaves may be advantageous for fending off insect attack as compared to the susceptible F1010 line. The BvSTI specific antibodies did not cross-react with F1016 and F1010 root proteins (data not shown) suggesting a possible high turnover, modification and/or non-detectable levels of BvSTI protein in the analyzed root samples regardless of the observed transcript levels (Fig. 2a, b). Lack of detection of recombinant proteins in transgenic plants that exhibited increased insect resistance has been reported previously (Maheswaran et al. 2007). But it has also been shown that increases in inhibitor protein concentrations did not proportionately increase insect resistance (Tamhane et al. 2007). Low concentrations of recombinant CamPI protein sufficiently inhibited up to 70 % of gut proteinase activity of *H. armigera* with no further increase of inhibition as the protein accumulated (Tamhane et al. 2007).

Comparison of trypsin inhibitor activities in non-wounded and wounded roots and leaves revealed a strong increase in activities in response to mechanical wounding in the resistant and susceptible line (Fig. 4). However, the 30 kDa BvSTI PI protein activity was not observed in F1010 roots either before or after wounding. This suggests that in the susceptible F1010 roots, the expected BvSTI protein was not present or was completely inactive despite the observed root transcript levels (Fig. 2b). The complete lack or reduced accumulation of active BvSTI protein in root maggot target tissues (i.e. roots) strongly emphasizes the possible role of the *BvSTI* gene in SBRM resistance. In gel trypsin inhibitor activity results revealed one more interesting point. The 50 kDa trypsin inhibitor band detected in the resistant F1016 line was never detected in any of the F1010 tissues suggesting that this unidentified protein may be

associated with defense against insect pests that utilize trypsin proteases to digest plant tissues.

We conclude that the levels of *BvSTI* gene activity in mechanically wounded sugar beet lines used in breeding for root maggot resistance coincide with their reported resistance status. Similarly, the observed lack or reduced accumulation and activity of BvSTI PI in tissues of the susceptible F1010 line emphasize the potentially important role of BvSTI PI in insect pest defense machinery. This data can lead to the use of *BvSTI* as a molecular marker in sugar beet breeding programs and in evaluating germplasm for resistance. A marker test, preferably relying on PCR, could be developed that would distinguish between resistant and susceptible beets. Mapping the respective genes would also provide more significant information if PI gene map positions coincide with a pest resistance QTL. A transgenic approach based on the expression of the *BvSTI* gene or its expression in combination with other resistance genes may also prove effective for improving insect resistance in elite sugar beet germplasm or other crops that would benefit from improved insect resistance.

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